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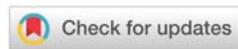
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Research Article

In-vitro susceptibility of wound bacteria of domestic carnivores to different mixtures of honey and *Nigella sativa* L. seed extracts

Abstract

The aim of this study is to evaluate the bactericidal activity of honey alone and honey mixed with different *Nigella sativa* L. seed extracts against bacterial isolates from chronic wounds of domestic carnivores. The preparation of *Nigella sativa* L. seed extracts was carried out by macerating the seed powder in increasingly polar solvents (ethyl acetate, ethanol and methanol). The study of the minimum bactericidal concentration of honey alone and honey mixed with different *Nigella sativa* L. seed extracts was carried out by Broth Dilution Method. The results showed that the addition of different *Nigella sativa* L. seed extracts decreased the bactericidal activity of honey against *Escherichia coli* and potentiated this bactericidal activity against *Staphylococcus intermedius*. Ethanolic and ethyl acetate extracts of *Nigella sativa* L. seeds did not influence the bactericidal activity of honey against *Staphylococcus aureus* and *Enterococcus faecalis*. Addition of different *Nigella sativa* L. seed extracts showed a variable influence on the bactericidal activity of honey against *Enterobacter sp.*. Methanolic extract of *Nigella sativa* L. seeds potentiated the bactericidal activity of honey against *Staphylococcus aureus*, *Staphylococcus intermedius*, *Enterococcus faecalis* and *Enterobacter sp.*. These results revealed that combinations of methanolic extract of *Nigella sativa* L. seeds with honey can be used for the development of potent antibacterial dressing.

Abreviations

BEA: Bile Esculin Azide; CFU: Colony-Forming Unit; g: gramme; ml: milliliter; MBC: Minimum Bactericidal Concentration; NM: Natural Mixture; PBS: Phosphate Buffered Saline; TQ: Thymoquinone; v/v: volume/volume; w/w: weight/weight; µl: microliter; %: percentage; °C: Degree Celsius;

Introduction

The skin of domestic carnivores is subjected to incessant aggression, trauma causing cutaneous wounds, which is one of the first reasons for consultation in veterinary medicine. The treatment of wounds aims to avoid complications and to obtain a quality healing as fast as possible. The physiological process that allows tissue restoration is wound healing. Infection is one of the factors that hinder this process. Since the use of antibiotics spread more than 50 years ago, bacteria have gradually developed resistance that continues to be a major health problem worldwide [1]. The use of natural products as an alternative treatment in wound healing and treatment has been on the rise in the last few decades [2].

Nigella sativa L. and honey are the most important natural remedies of Arabic Islamic medicine and used for various diseases for over 1500 years. In Islam, *Nigella sativa* L. and honey are regarded as one of the greatest forms of healing medicine [2]. The ancient Greeks and Egyptians used topical application of honey to treat wounds and burns of the skin, and traditional Persian medicine has documented that honey is effective in treating wounds, eczema and inflammation [3, 4]. Scientific interest in honey decreased with the advent of antibiotics in the early 1900 years [5], but today with the emergence of antibiotic resistant microbial strains, honey has again attracted the attention of researchers [5, 6]. Honey has long been known to possess antibacterial properties and has established use as a dressing [7,8], but not all honeys are equally effective in wound healing [9]. In human medicine, several published reports indicate the efficacy of honey in the treatment of various known infected wounds such as venous ulcers in the leg [10,11], burns [12], chronic leg ulcers [13] and pressure ulcers [14]. *Nigella sativa* L. has traditionally been used for centuries in the Middle East, North Africa, the Far East and Asia for the treatment of various diseases [15], as well as in the treatment of many types of wounds and trauma [16,17].

In this research, the bactericidal activity of Algerian honey against bacterial isolates from chronic wounds of domestic carnivores, was evaluated. Also, influence of addition (5% w/w) of different *Nigella sativa* L. seed extracts on the bactericidal activity of this honey was studied. Honey and *Nigella sativa* L. seed extracts were combined together for the inhibition of bacterial growth to evaluate the synergistic effect of this combination on these bacteria. Different extracts of *Nigella sativa* L. seeds were used for this combination to determine the extract capable of giving the most powerful synergistic effect with honey. These extracts were used with a low percentage so as not to alter too much the physical and chemical properties of the honey.

Materials and Methods

Plant material

The seeds of *Nigella sativa* L. were purchased from an herbal shop in El-Eulma, Algeria.

Honey sample

The honey used in this study was purchased from an apiary in El-Eulma, Algeria.

Bacterial isolates

Five bacteria were isolated from chronic wounds of dogs and cats, using three selective media (Chapman, MacConkey and BEA). These bacteria were identified by standard and biochemical bacteriological tests and were maintained at 4°C on nutrients agar slants.

Extraction process

The seeds of *Nigella sativa* L. were cleaned of impurities, washed and dried away from light for a few days, and then ground with a mortar to medium fine powder from which different extracts were prepared.

The extraction was carried out by macerating the seed powder in increasingly polar solvents (ethyl acetate, ethanol and methanol) following the method described by Shahid *et al.* (2013) [18].

Each of 50 g seed powder was macerated in 500 ml of a different solvent, for two weeks at room temperature, with occasional agitation to facilitate extraction. The macerates were filtered on filter papers. The filtrates were evaporated using rotary evaporator (Heidolph®) at 50°C. The extracts were stored in sterile glass vials at 4°C until use.

Preparation of natural mixtures

Three natural mixtures were prepared by adding 5% (w/w) of different *Nigella sativa* L. seed extracts to honey. The composition of these natural mixtures was:

- The first natural mixture (NM 1): 95% of honey + 5% of ethyl acetate extract;
- The 2nd natural mixture (NM 2): 95% of honey + 5% of ethanolic extract;

- The 3rd natural mixture (NM 3): 95% of honey + 5% of methanolic extract.

All natural mixtures were freshly prepared and stored in the refrigerator at 4°C in the dark.

Inoculum preparation

The inoculums was prepared following the method described by Majtan *et al.* (2010) [19] with small changes in the use of nutrient broth instead of PBS.

For each bacterial strain, an active culture on nutrient agar was carried out. Then a few colonies of this culture were suspended in nutrient broth. With a spectrophotometer, the turbidity of the bacterial suspension was adjusted to 10⁸ CFU/ml and diluted with nutrient broth to final concentration of 10⁷ CFU/ml.

Study of the bactericidal activity of honey alone and natural mixtures

The study of the bactericidal activity of honey alone and natural mixtures (NMs) was carried out by the Broth Dilution Method following the protocol described by Alzahrani *et al.* [20], with some modifications.

For honey alone and for each natural mixture, 14 sterile test tubes were used to test different concentrations (v/v): 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, 0.39%, 0.19%, 0.09%, 0.04%, 0.02% and 0.01%.

Using a sterile graduated pipette, 1 ml of honey alone and 1 ml of each natural mixture was transferred separately into the first test tubes of the concentration 100% (no dilution). 1 ml of Muller-Hinton broth was transferred into each of the other test tubes for other decreasing concentrations. Then, 1 ml of honey alone and 1 ml of each natural mixture was transferred separately into the first test tubes containing Muller-Hinton broth to obtain a concentration of 50%. The contents of these test tubes were homogenized by a vortex. After homogenization, 1 ml of the contents of each test tube of the concentration 50% was transferred into the next test tube. The same procedures were repeated until the concentration 0.01% from which, after good homogenization, 1 ml of the test tube contents was discarded.

10 µl (10⁵ CFU) of each standardized bacterial suspension was poured into the test tubes of different concentrations. A positive control was made by inoculating 10 µl (10⁵ CFU) of each standardized bacterial suspension into 1 ml of Muller-Hinton broth. Negative control was made by adding 0.5 ml of Muller-Hinton broth to 0.5 ml of honey alone and 0.5 ml of each natural mixture. All test tubes were incubated at 37°C for 24 hours.

For each test tube, after incubation, subculture was done after good homogenization by inoculating 10 µl of culture medium (contained in the test tubes) onto the surface of nutrient agar spread out in Petri dishes. After incubation at 37°C aerobically for 24 hours, the number of bacterial colonies of the sub-cultured nutrient agar was calculated. The MBC was

determined as the lowest concentration showing no bacterial growth.

All tests were performed in triplicate and were repeated three times to obtain reliable results.

Results and Discussion

The results concerning the standard and biochemical

bacteriological tests of isolated bacteria are gathered in table 1. These bacterial isolates are: *Escherichia coli*, *Enterobacter sp.*, *Staphylococcus aureus*, *Staphylococcus intermedius* and *Enterococcus faecalis*.

The results concerning the bactericidal activity of honey alone and natural mixtures against the bacterial isolates are gathered in table 2. Also, the results concerning the MBC of these natural products are gathered in table 3.

Table 1: Results of standard and biochemical bacteriological tests of isolated bacteria.

Strains	Culture Media	Macroscopic appearance of colonies	Gram stain	Catalase test	Oxidase test	API 20E	API Staph	API 20Strep
Strain n° 01	MacConkey	Pink colonies (lactose +)	Gram- rods	/	(-)	<i>Escherichia coli</i>	/	/
Strain n° 02	Chapman	Golden yellow colonies (mannitol +)	Gram+ cocci in clusters resembling bunches of grapes	(+)	/	/	<i>Staphylococcus aureus</i>	/
Strain n° 03	BEA	Translucent colonies with a black halo	Gram+ cocci in chains	(-)	/	/	/	<i>Enterococcus faecalis</i>
Strain n° 04	MacConkey	Yellow colonies (lactose -)	Gram- rods	/	(-)	<i>Enterobacter sp.</i>	/	/
Strain n° 05	Chapman	Pink colonies (mannitol -)	Gram+ cocci in clusters resembling bunches of grapes	(+)	/	/	<i>Staphylococcus intermedius</i>	/

(+): positive reaction. (-): negative reaction.

Table 2: The bactericidal activity of honey alone and natural mixtures (NMs) against the bacterial isolates.

Concentration of natural product (dilution v/v)	Natural product	Number of colonies of bacterial strains				
		<i>Escherichia coli</i>	<i>Enterobacter sp.</i>	<i>Staph. aureus</i>	<i>Staph. intermedius</i>	<i>Enterococcus faecalis</i>
100%	NM 1	-	-	-	-	-
	NM 2	-	-	-	-	-
	NM 3	-	-	-	-	-
	HA	-	-	-	-	-
50%	NM 1	-	-	-	-	-
	NM 2	-	-	-	-	-
	NM 3	-	-	-	-	-
	HA	-	-	-	-	-
25%	NM 1	-	+++	+++	-	+++
	NM 2	-	-	+	-	+++
	NM 3	-	-	-	-	-
	HA	-	-	++	-	+
12.5%	NM 1	+++	+++	+++	-	+++
	NM 2	+++	+++	+++	-	+++
	NM 3	+	-	-	-	-
	HA	-	+++	+++	+++	+++
6.25%	NM 1	+++	+++	+++	-	+++
	NM 2	+++	+++	+++	-	+++
	NM 3	+++	+++	+++	++	+++
	HA	+++	+++	+++	+++	+++
3.12%	NM 1	+++	+++	+++	+++	+++
	NM 2	+++	+++	+++	+++	+++
	NM 3	+++	+++	+++	+++	+++
	HA	+++	+++	+++	+++	+++

NM: natural mixture. HA: honey alone. (-): no growth. (+): 1-10 bacterial colonies. (++) : 10-100 bacterial colonies. (+++) : >100 bacterial colonies.

Note: For other concentrations (1.56% - 0.01%), the number of colonies of all the bacterial strains was >100 (+++).

Table 3: The MBC of honey alone and natural mixtures (NMs) against the bacterial isolates.

Natural product	Minimum bactericidal concentration (MBC) of natural products				
	<i>Escherichia coli</i>	<i>Enterobacter sp.</i>	<i>Staph. aureus</i>	<i>Staph. intermedius</i>	<i>Enterococcus faecalis</i>
NM 1	25%	50%	50%	6.25%	50%
NM 2	25%	25%	50%	6.25%	50%
NM 3	25%	12.5%	12.5%	12.5%	12.5%
HA	12.5%	25%	50%	25%	50%

NM: natural mixture. HA: honey alone.

For *Escherichia coli*, the bactericidal activity of honey alone was the highest since it killed all the inoculums with a concentration of 12.5% which was the MBC. The MBC of natural mixtures was 25%. NM 3 killed almost all the inoculums with a concentration of 12.5% and only a few colonies (1-10 colonies) appeared on the nutrient agar after 24 hours of incubation.

For *Enterobacter sp.*, the bactericidal activity of NM 3 was the highest since it killed all the inoculums with a concentration of 12.5% which was the MBC. The MBC of honey alone and NM 2 was 25%, and that of NM 1 was 50%. This latter natural mixture had the lowest bactericidal activity.

For *Staphylococcus aureus*, the bactericidal activity of NM 3 was the highest with a MBC of 12.5%. The MBC of all other natural products was 50%. NM 2 killed almost all the inoculums with a concentration of 25% and only a few colonies (1-10 colonies) appeared on the nutrient agar after 24 hours of incubation.

For *Staphylococcus intermedius*, the bactericidal activity of NM 1 and NM 2 was the highest with a MBC of 6.25%. The MBC of NM 3 and honey alone were 12.5% and 25% respectively. The bactericidal activity of honey alone was the lowest.

For *Enterococcus faecalis*, the bactericidal activity of NM 3 was the highest with a MBC of 12.5%. The MBC of all other natural products was 50%. Honey alone killed almost all the inoculums with a concentration of 25% and only a few colonies (1-10 colonies) appeared on the nutrient agar after 24 hours of incubation.

These results showed that addition of some *Nigella sativa* L. seed extracts may potentiate the bactericidal activity of honey. There seems to be a synergism between honey and some extracts of *Nigella sativa* L. seeds according to the type of added extract and the tested bacterium. The exact mechanisms of synergy between *Nigella sativa* L. seed extracts and honey are uncertain and further studies are needed to elucidate these mechanisms.

Also, these results showed that addition of some *Nigella sativa* L. seed extracts may decrease the bactericidal activity of honey against certain bacteria. This result may be the consequence of the modification of the physical and chemical properties of honey and further research studies could elucidate this statement.

NM 3 showed the highest bactericidal activity since it had the smallest MBC against *Enterobacter sp.*, *Staphylococcus aureus* and *Enterococcus faecalis*. This natural mixture killed almost all

the inoculums of *Escherichia coli* with a concentration of 12.5% and only a few colonies (1-10 colonies) appeared on the nutrient agar after 24 hours of incubation. Also, this natural mixture was more effective than honey alone against *Staphylococcus intermedius*.

The antimicrobial property of honey depends on several contributing factors. It can be the result of high sugar concentration, acidity, production of hydrogen peroxide, flavonoids, phenols or other unidentified components present in honey [21]. The phenolic compounds, particularly the flavonoids, are responsible for the antimicrobial properties of honey [22-24]. Certain types of honey contain other bioactive components with antibacterial activity, including methylglyoxal, lysozyme and defensin-1 [25,26]. In addition, it is suggested that presence of different strains of *Lactobacillus acidophilus* in honey obtained from different sources may contribute to the antimicrobial properties of honey [27]. Also, hydrogen peroxide plays an important role in the antimicrobial activity of honey [28].

The ethanolic, methanolic and ethyl acetate extracts of *Nigella sativa* L. seeds are rich in TQ [29,30] which has a powerful antibacterial effect [31-33].

Clinical trials using NM 3 to treat animal wounds could further confirm the findings of this work. Further research is needed to elucidate and optimize the effective combination of these natural products in clinical practice.

Management of the animal wounds infection should not be limited to local wounds care but may involve other modalities including systemic antibiotics and debridement whenever needed. Neither of these natural products has an adverse effect on tissues, so they can safely be used on animal wounds to clear infection.

Conclusion

The synergism between Algerian honey and methanolic extract of *Nigella sativa* L. seeds can be exploited for the treatment of infected wounds and other bacterial infection. This synergistic antibacterial effect can be extremely useful in treatment of infected wounds of domestic carnivores.

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